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Renal tubular injury induced by hyperoxaluria: evaluation of apoptotic changes

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Abstract In order to evaluate the injurious effect of hyperoxaluria on renal tubular epithelium, as judged by apoptotic changes in the renal parenchyma, we performed an experimental study in 20 rabbits. In the experimental group animals ($n = 10$) severe hyperoxaluria was induced by continuous ethylene glycol (EG; 0.75%). Histologic alterations, including crystal formation, together with apoptotic changes were evaluated after 7 and 28 days. Control group animals ($n = 10$) received normal distilled drinking water. Following 7- and 28-day periods, tissue sections obtained from kidneys were examined histopathologically under light microscopy for the presence and the degree of crystal deposition in the tubular lumen. Apoptotic changes in renal tubular cells were examined using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick and labeling (TUNEL) method during the same follow-up period. Crystal deposition was evident in the tubular lumen of tissue sections obtained during the 7-day examination period. During the 28-day examination period, however, these findings were found to be either limited or to have disappeared. In relation to apoptotic changes, the percentage of positive nuclei stained using the TUNEL method was from 11 to 20% in the experimental group and 5.6% in the control group. Our findings indicate that both calcium oxalate (CaOx) crystals and hyperoxaluria itself may be injurious to renal tubular cells, as indicated by apoptotic changes. These changes may be responsible for the pathologic course of urolithiasis.

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Introduction

Previous studies have pointed out that hyperoxaluria is the main risk factor for human idiopathic calcium oxalate (CaOx) stone formation and that the induction of hyperoxaluria is essential for the development of CaOx urolithiasis [4–6, 12]. Related to this subject, the interaction between renal epithelial cells and CaOx crystals and/or oxalate ions plays a critical role in the formation of urinary calculi [3, 7, 13]. Although crystals themselves can cause cell damage, the cause-and-effect relationship between injury and crystals has not yet been elucidated. Both clinical and experimental studies have suggested that the renal tubular epithelium is the major target for oxalate-induced injury and that sustained hyperoxaluria and CaOx crystal formation/deposition may result in injury to renal tubular cells [3, 7, 13].

Taking the results of recent studies on LLC-PK1 cells into account, oxalate exposure has been found to produce a variety of changes in renal epithelial cell morphology and function, including increased cellular proliferation and, at elevated concentrations, cell death. The induction of hyperoxaluria has been found to be associated with necrosis and cell injury in the renal tubular epithelium. These and other studies demonstrate that the interaction of oxalate with renal epithelial cells elicits a programmed sequence of events that can lead to either cell proliferation or cell death [11, 15, 17, 19]. Thus, a high oxalate concentration together with CaOx crystal formation/deposition may induce renal tubular cell damage and/or dysfunction, which may express itself as cell apoptosis.

In the present animal study, our main goal was to evaluate the presence and degree of apoptotic changes in renal tubular epithelial cells, following hyperoxaluric diet induction in a rabbit model.

Materials and methods

The experimental animals were male New Zealand white rabbits, each weighing 3–5 kg. All animals were fed standard chow and kept in normal room conditions. Before any medication was administered or the hyperoxaluric diet started, all animals underwent a thorough evaluation with respect to anatomic or biochemical abnormalities in the Department of Pathology. Following a thorough physical examination, all animals underwent biochemical evaluation, including blood and urine analyses and stool examination. Ultrasonographic examination of the kidneys was performed to detect any anatomic abnormalities. No pathologic finding or urinary tract infection could be shown during these evaluations.

The rabbits were divided into two groups. Group I: the experimental animals ($n = 5$ for the 7-day time period, and $n = 5$ for the 28-day period) were given a hyperoxaluria-inducing diet of 0.75% ethylene glycol (EG) in distilled drinking water for a 2-week period before undergoing specific examination after 1 and 4 weeks, respectively (depending on which time period they were in). Group II: the control animals ($n = 5$ for the 7-day time period, and $n = 5$ for the 28-day period) received distilled drinking water. During the experimental period all animals were provided regular rabbit chow ad libitum. Following a 2-week period of hyperoxaluric diet in group I and normal diet in group II, animals were killed after 1 or 4 weeks. Bilateral flank incision was performed, and the kidneys were removed for histopathologic evaluation.

Evaluation of renal tissue histology and crystal deposition was performed under light microscopy. Tissue specimens were fixed with 10% formalin solution and embedded in paraffin blocks. After this procedure, 3–5 μm sections were obtained with a microtome for hematoxylin and eosin (H&E) staining. The tissue sections were examined under a light microscope (10×40, 10×10). Utilizing a specific grading system, tubules demonstrating granular crystallization and/or calcification were counted in an area of 1 cm^2, and the extent of the crystallization was graded as follows: minimal (+) = crystallization and/or calcification in 1–3 tubules; moderate (++) = same findings in 4–7 tubules; and severe (+++) = same findings in 7 or more tubules.

In situ detection of apoptosis

The in situ detection of renal tubular cells with DNA strand breaks in paraffin-embedded parenchymal sections was achieved with the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP in situ nick and labeling (TUNEL) method using an ApopTag kit (Oncor, Gaithersburg, Md., USA). Briefly, after deparaffinization and rehydration, tissue sections were incubated with proteinase K (200 μg/ml; Oncor) for 20 min at room temperature, washed in distilled water, and then treated with 3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min at room temperature to quench endogenous peroxidase activity. Sections were incubated with terminal deoxynucleotidyl transferase (TdT) and dioxigenin-1 l-dUTP in a humidified chamber at 37 °C for 1 h, and then treated with antidioxigenin-peroxidase at room temperature for 30 min. Subsequently, sections were exposed to 0.05% substrate for 7 min, washed with distilled water and PBS, and then counterstained for 10 min. They were then dehydrated in 100% butanol, cleared in xylene, and mounted with Entellan (Merck Scientific, Fairlacon, N.J., USA). Negative controls were obtained by omitting the TdT enzyme and using the same volume of distilled water. The ApopTag kit used during the study contained the positive controls.

Quantification of apoptosis in tubular cells

To quantitate the incidence of apoptosis at each time point, the percentage of TUNEL-positive cell nuclei within renal tubule cross-sections was calculated after counting 3,000 tubular cells in each preparation.

Results

Light microscopy findings

Examination of renal parenchymal tissue subjected to a hyperoxaluric diet for 2 weeks revealed various degrees of crystal deposition, which was observed mainly in the tubular lumen. Apart from a slight degree of crystal deposition, no notable histologic alteration could be demonstrated in interstitial areas. Deposition in the kidneys was widespread in two (Fig. 1), moderate in two, and minimal or slight in one during a 1-week follow-up examination. Crystal deposition tended to limit itself during long-term (4 weeks) evaluation; it was found to be moderate in two kidneys and minimal in one kidney.

On the other hand, evaluation of tissue specimens from control group animals receiving normal distilled water revealed no detectable crystal formation/deposition in any of the specimens examined, as expected.

Evaluation of apoptotic changes

The quantitative evaluation of TUNEL-positive cell nuclei in tissue sections obtained from both animal groups revealed the following findings. A large number of positively stained nuclei were present after 1 week. The percentage of positively stained nuclei in crystal-forming rat kidneys varied from 11 to 20%. The degree of apoptosis was found to be prominent as the rate of crystal deposition increased. In other words, more apoptotic changes were present close to crystal deposits (Fig. 2). Thus, in addition to hyperoxaluria itself, crystal formation and deposition also affected the degree of apoptosis in tubular cells. Apoptotic changes have been found to be more prominent in tubular cells surrounding the crystals; however, some degree of apoptotic changes could also be noted in tubular cells even when no crystal

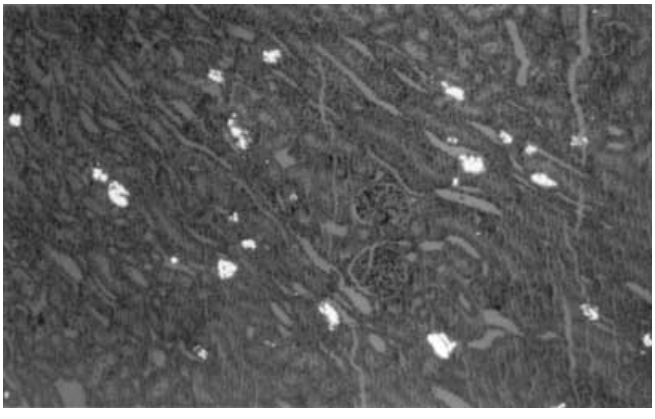

Fig. 1 Severe (+++) crystal deposition observed in a cross-section of the kidney obtained from the hyperoxaluria group during 1-week follow-up (polarized light microscopy, ×100)

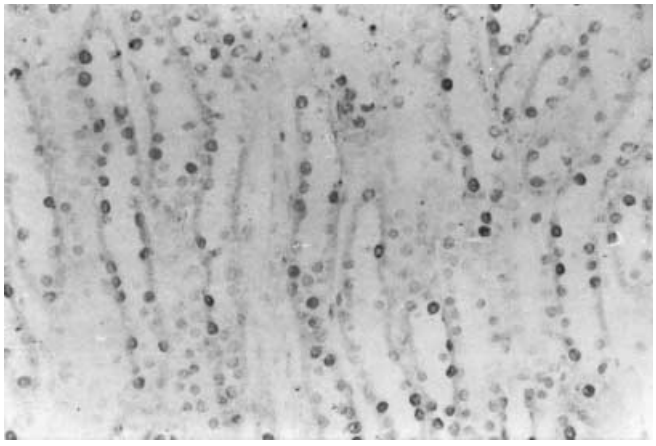

Fig. 2 Evident apoptotic changes could be seen from a cross-section of the kidney obtained from the hyperoxaluria group during 1-week follow-up. TUNEL-positive cell nuclei are stained black (*arrows*) (TUNEL method)

formation could be seen. On the other hand, while apoptotic changes were prominent in distal tubular cells, as well as in collecting duct cells, the degree of these changes was less prominent in proximal tubular cells.

Despite widespread apoptotic changes noted during early follow-up, evaluation of the sections obtained from hyperoxaluric rabbits during long-term (4 weeks) examination demonstrated limited or no TUNEL positivity in the majority of tubules (Fig. 3). Apoptotic changes tended to be limited, being observed in only a small number of tubules. The percentage of positively stained nuclei varied from 6.5 to 9.6%. In addition to apoptotic changes, crystal deposition also decreased in the majority of tubules; it did, however, persist to some extent.

Evaluation of tissue sections obtained from control animals did not demonstrate any significant evidence of apoptotic changes in tubular epithelial cells. The percentage of TUNEL-positive nuclei varied from 0 to 4.5% during 1 week and from 0 to 2.8% during long-term evaluation; in addition, positively stained cell nuclei were present in a very limited number of tubules. The degree of crystal deposition, as well as apoptosis, in both groups is demonstrated in Table 1.

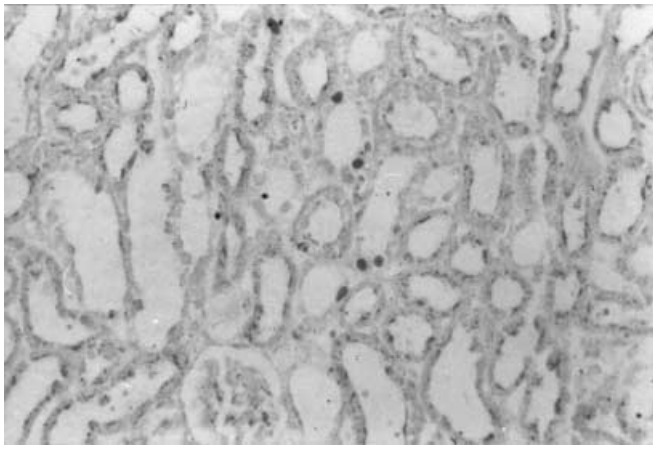

Fig. 3 Limited apoptotic changes could be seen in tubular cells in the cross-section of a kidney obtained from the hyperoxaluria group during 1-month follow-up (TUNEL method)

Discussion

It has been recognized that hyperoxaluria is the main risk factor for idiopathic CaOx stone formation and that the induction of hyperoxaluria is crucial for the development of CaOx urolithiasis [2, 5, 7, 12]. Experimental studies demonstrated that the interaction between renal epithelial cells and CaOx crystals and/or oxalate ions plays a critical role in the formation of urinary stones. Related to the pathogenesis, sustained hyperoxaluria and subsequent CaOx crystal formation/deposition may result in injury to the renal tubular cells. This will eventually result in cell death and sloughing, exposing the basement membrane. On the other hand, the induction of hyperoxaluria has been found to be associated with cellular injury and necrosis in tubular epithelial cells [5–8, 13]. Demonstration of enzymuria, proteinuria and membranuria following hyperoxaluric diet in animal models also supports the proposal that hyperoxaluria, either itself or in combination with CaOx crystal formation, could be injurious to renal tubular epithelial cells [10].

Moreover, all of these changes, i.e., injury to the tubular cells, were observed even in the absence of crystalluria, suggesting that oxalate-induced damage is not solely the result of injury produced by CaOx crystals [9, 12, 14]. Regarding the etiopathogenesis of cell injury during hyperoxaluric diet, in their original study, Thamilselvan et al. [20] demonstrated induced lipid peroxidation in tubular cells, which usually points to functional impairment of the cellular components by reactive oxygen species (ROS) [1, 18, 20]. In their original study, Scheid et al. [17] demonstrated that, of all the various mono- and dicarboxylates examined in their study, oxalate was the most potent at increasing the level of free radical production and cell death. They proposed that oxalate may increase the availability of free radicals by inhibiting enzymes responsible for their degradation

Table 1 Evaluation of the degree of crystal deposition, as well as apoptosis, in control and hyperoxaluria-treated kidneys: cross-sections at each time point (percentage values given in parentheses). + Minimal (1–3 tubules in each cross-section); + + Moderate (4–7 tubules in each cross-section); + + + Severe (7 or more tubules in each cross-section)

Days	Hyperoxaluria			Control		
	+	+ +	+ + +	+	+ +	+ + +
7	1/5 (11)	2/5 (15)	2/5 (20)	–	–	–(0–4.5)
28	1/5 (6.5)	2/5 (9.6)	–	–	–	–(0–2.8)

[16]. Apart from possible tubular ischemia, which gives rise to ROS production as a result of lipid peroxidation, the results of some animal studies indicate that the interaction of oxalate ions with renal epithelial cells may initiate a programmed sequence of events that can lead to cell proliferation or cell death – in other words, the possible involvement of cell apoptosis in cellular death [11, 15, 17, 19]. In their original study, Koul et al. [12] showed that there may be a causal link between the oxalate-induced increase in *C-myc* gene expression and the oxalate-induced increase in cellular proliferation [19]. Taking the results of recent studies on LLC-PK1 cells into account, oxalate exposure has been found to produce a variety of changes in renal epithelial cell morphology and function, including increased cellular proliferation and, at elevated concentrations, cell death. The induction of hyperoxaluria has been found to be associated with necrosis and cell injury in the renal tubular epithelium. These and other studies demonstrate that the interaction between oxalate and renal epithelial cells elicits a programmed sequence of events that can lead to either cell proliferation or cell death [11, 15, 17, 19]. Thus, as a distinctive form of cell death, apoptosis could also be responsible for the injury induced by hyperoxaluria.

In this experimental study, our aim was to evaluate the presence and degree of apoptotic changes during hyperoxaluria. Animals fed a hyperoxaluric diet during the course of the study program were found to demonstrate significant crystal formation/deposition together with apparent TUNEL-positive cell apoptosis in the early phase of follow-up (1 week). Apoptotic changes were widespread and evident in animals forming a higher degree of CaOx crystals in the tubules. Quantitative examination of cell apoptosis has also demonstrated the predominance of tubular cell apoptosis in early follow-up. However, both apoptotic changes and crystal deposition tended to decrease in late follow-up examination (4 weeks). Although apoptotic changes either disappeared or became limited to a small number of tubules, crystal deposition to a certain extent persisted in some tissue sections after 4 weeks, even though the hyperoxaluric diet had been discontinued. No crystal deposition together with insignificant apoptotic changes could be demonstrated in control group animals.

Our findings indicate the occurrence of cell apoptosis during a hyperoxaluric diet, especially during the early phase of follow-up. These alterations tended to disappear to a significant extent over time, which may in turn indicate an adaptation reaction of the body itself. This is possibly the result of the proliferation process beginning during late follow-up. In light of our findings and the literature data as well, we may say that both oxalate and CaOx crystals are injurious to renal epithelial cells. Apoptotic changes observed in renal tubular epithelial cells damaged by massive hyperoxaluria may be responsible for the pathologic course of urolithiasis.

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